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U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

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INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR00/01173	October 18, 2000	April 8, 2000

TITLE OF INVENTION

A TRANSFORMANT FOR SCREENING OF INHIBITORS FOR HUMAN IMMUNODEFICIENCY VIRUS

EXPRESS MAIL CERTIFICATE
Date 12-6-01 Label No. EV004807013US

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

D Davis — Name (Print)

Signature

APPLICANT(S) FOR DO/EO/US

Hyuk-Jun NAM and Sang-Hern KIM

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 3 references and International Search Report).

12. [X] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [] A FIRST preliminary amendment.
    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [X] Other items or information:

- SEQUENCE LISTING (WRITTEN AND MACHINE-READABLE FORMS) with STATEMENT PURSUANT TO RULE 1.821(f)
- DECLARATION CONCERNING DEPOSITED MICROORGANISM UNDER BUDAPEST TREATY, WITH DEPOSIT RECEIPT
- AFFIRMATION OF PRIORITY CLAIM

JC10 Rec'd PCT/PTO 06 DEC 2001

APPLICATION NO. (if known see 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO.: PCT/KR00/01173	Attorney's Docket Number 0136/0K089US0

17. [x] The following fees are submitted:		CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [] or JPO []	$890.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$710.00		
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	$740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	$1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)....	$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$1,040.00	

Surcharge of $130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	20-**20**		X $18.00	$00	
Independent Claims	**8-3**		X $80.00	$400.00	
Multiple dependent claim(s) (if applicable)		+270		$	
			TOTAL OF ABOVE CALCULATIONS =	$1,440.00	
Reduction by 1/2 for filing by small entity, if applicable.				$720.00	
			SUBTOTAL =	$720.00	
Processing fee of $130.00 for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
			TOTAL NATIONAL FEE =	$720.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$40.00	
			TOTAL FEES ENCLOSED =	$760.00	
				Amount to be: refunded	$
				charged:	$

a. [X] A check in the amount of $760.00 to cover the above fees is enclosed.

b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

S. Peter Ludwig
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805 Third Avenue
New York, New York 10022-7513

SIGNATURE _____

NAME S. Peter Ludwig

REGISTRATION NO 25,351

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File No.: 0136/0K089US0

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Hyuk-Jun NAM and Sang-Hern KIM

Serial No: To Be Assigned (National Phase of International Application No. PCT/KR00/01173, filed October 18, 2000)

Filed: Concurrently Herewith

For: **A TRANSFORMANT FOR SCREENING OF INHIBITORS FOR HUMAN IMMUNODEFICIENCY VIRUS**

STATEMENT PURSUANT TO RULE 1.821(f)

December 6, 2001

Hon. Commissioner of Patents
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, Virginia 22202

Sir:

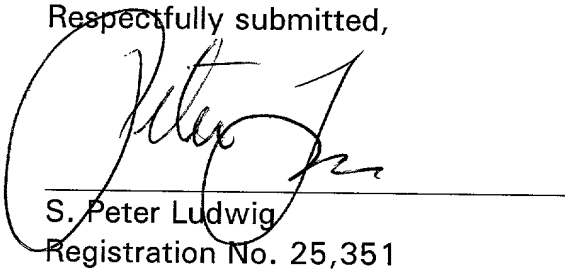
A diskette is enclosed which includes a sequence listing for the above-referenced application. A paper copy of the file is attached.

The sequence in the present Sequence Listing is shown in the specification of the corresponding international application no. PCT/KR00/01173 as published on October 18, 2001 and transmitted by the International Bureau; therefore, no new matter has been added as a result of the amendment filed herewith.

The content of the attached paper entitled "Sequence Listing" and of the accompanying machine-readable ASCII-encoded file on diskette labeled "Sequence list (ASCII Copy)", is the same. Furthermore, the information contained in the attached "Sequence Listing" and the ASCII-encoded file is identical to the information in the specification as filed.

Consideration of the enclosed diskette and paper are respectfully requested.

Respectfully submitted,



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M:\0136\0K089\EEB0607 WPD

A TRANSFORMANT FOR SCREENING OF INHIBITORS FOR HUMAN IMMUNODEFICIENCY VIRUS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a transformant for screening of human immunodeficiency virus("HIV") inhibitors, more particularly, to a transformant cotransformed with a plasmid expressing HIV nucleocapsid protein and a plasmid containing HIV psi(Ψ) nucleotide sequence and β-galactosidase reporter gene, and a method for screening of HIV inhibitors by employing the said transformant.

Background of the Invention

HIV, a pathogen causing the aquired immunodeficiency syndrome("AIDS"), selectively infects crucial immune cells called CD4+ T helper cells and replicates inside the cells. Infection of HIV leads to the lysis of CD4+ T cells resulting from an interaction between viral env glycoprotein and plasma membrane of target cell and a subsequent reproduction of virus particles. Also, the binding of soluble gp120 to CD4+ molecules onto uninfected T cells block interactions of CD4+ T cells with other immune cells. In addition to depleting CD4+ T cells, impaired are function of cytotoxic T cells expressing CD8+, antibody-dependent cytotoxicity, maturation of CD4+ T cells in thymus, interaction between CD4+ cells and class II MHC on antigen presenting cells, and function of macrophages and natural killer cells. Thus, human immune system is gradually deteriorated after HIV infection.

Until now, drugs suppressing HIV replication have been developed, which include reverse transcriptase inhibitors such as AZT(azidothymidine) and

ddI(dideoxyinosine), and protease inhibitors. Recently, the researches to develop DNA vaccines employing nucleotide sequence encoding HIV proteins(see: Hinkula J. et al., Vaccine, 15:874-878, 1997; Calarota et al., Lancet, 351:1320-1325, 1998), and live-attenuated HIV vaccines made by deleting the HIV nef gene are being undertaken(see: Kestler and Jeang, Science, 270:1219-1222, 1995; Chakrabarti et al., Proc. Natl. Acad. Sci., USA, 93:9810-9815, 1996).

Since the said drugs are not able to remove the provirus of HIV of which DNA is inserted into the host immune cell chromosome or not able to selectively remove host immune cells containing the provirus, it cannot be excluded that HIV variants arisen by genetic mutation acquire drug resistance or HIV revertants arisen by recombination of attenuated virus vaccine acquire characteristics of pathogenic HIV(see: Berkhout et al., J. Virol., 73:1138-1145, 1999). Furthermore, it has been found that HIV requires not only CD4+ molecule as the receptor on the surface of host cells, but also coreceptors such as 'T-cell-line-tropic' CXCR4/fusin coreceptor or 'macrophage-tropic' CCR5 coreceptor for its binding and gaining entry of HIV into host cells(see: Feng et al., Science, 272:872-877, 1996). Thus, one approach for drug therapy is to target these coreceptors in an attempt to inhibit binding of virus onto the host cells. Since the normal function of these coreceptors is to bind 'chemokines(chemotactic cytokines)' which plays a role in inflammation reaction, serious side effects may be anticipated(see: Murphy, P.M., Ann. Rev. Immunol., 12:593-633, 1994). In view of above situation, there is a need to develop a novel class of HIV inhibitors which do not affect host immunity or physiological activity relating to receptors, and one approach for such drug therapy is to target HIV specific factors required for virus assembly.

When HIV virus particle is assembled, its genomic RNA is selectively packaged into a virion. It is well known

that a specific interaction between the region of nucleocapsid(NC) protein and the viral packaging sequence(encapsidation signal), psi(Ψ), allows selective packaging of viral genomic RNA. Psi(Ψ), located between long terminal repeat(LTR) of 5'-terminal of genomic RNA and *gag* gene which encodes precursor poly protein(matrix-capsid-nucleocapsid), has 4 stem-loop structures. However, the screening of compounds or compositions which inhibit the specific viral interaction required for packaging is difficult due to the lack of an easy and efficient assay system for such screening.

Under the circumstances, there are strong reasons for exploring and developing a model system which can be used to detect the specific interaction between HIV NC protein and HIV psi(Ψ) sequence as in *in vivo*, for screening of inhibitors against HIV packaging.

Summary of the Invention

The present inventors have made an effort to develop a simple and effective method for detecting the specific interaction between HIV NC protein and HIV psi(Ψ) sequence in vivo to screen HIV packaging inhibitors, thus, prepared transformants cotransformed with a plasmid expressing HIV NC protein and a plasmid containing HIV psi(Ψ) sequence and β-galactosidase reporter gene, and found that HIV inhibitors can be conveniently screened by employing the said transformant on the basis of the expression level change of β-galactosidase.

A primary object of the invention is, therefore, to provide a transformant cotransformed with a plasmid expressing HIV nucleocapsid protein, and a plasmid containing HIV psi(Ψ) sequence and β-galactosidase reporter gene.

The other object of the invention is to provide a method for screening HIV packaging inhibitors employing the

said transformant.

BRIEF DESCRIPTION OF THE DRAWINGS

The above, the other objects and features of the invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1a is a schematic representation of construction strategy of pX1(-ATG).

Figure 1b is a schematic representation of construction strategy of pNH1.

Figure 1c is a schematic representation of construction strategy of pNH1Psi or pNH1rePsi.

Figure 2 is a graph showing the expression of β-galactosidase in *E. coli* JM109 cotransformed with each of pJC1 expressing nucleocapsid protein, or pTrcHisGag expressing Gag protein, or pSE380 as a control plasmid, and pNH1, respectively.

Figure 3a is a graph showing the effect of interaction between HIV nucleocapsid protein or Gag protein and HIV psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in *E. coli* JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1MCS, respectively.

Figure 3b is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and HIV psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in *E. coli* JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1Psi(SL1234), respectively.

Figure 4 is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and various portions of psi(Ψ) sequence on the expression of β-galactosidase in *E. coli* JM109 which is cotransformed with each of pJC1, pTrcHisGag or pSE380 and each of plasmid pNH1Psi(SL1234), pNH1Psi(SL234), pNH1Psi(SL34), pNH1Psi(SL23), or pNH1Psi(SL12) containing psi(Ψ) nucleotide sequence, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The invented transformant employed for screening of HIV packaging inhibitors is prepared by cotransforming a plasmid pJC1 expressing HIV NC protein and a plasmid pNH1Psi(SL1234) containing HIV psi(Ψ) gene and β-galactosidase reporter gene.

The process for preparing the transformant for screening of HIV inhibitors is further illustrated in more detail.

The transformant was prepared by cotransforming with pJC1 expressing HIV NC protein and pNH1Psi(SL1234) containing HIV psi(Ψ) gene and β-galactosidase reporter gene: pX1(-ATG) was constructed by removing ATG from pX1 plasmid which was made by inserting lacZ gene fragment into pSE280. And then, pZ1 was obtained by substituting ampicillin resistance gene of pX1(-ATG) with the AflIII-StuI fragment containing kanamycin resistance gene. pNH1 containing lacZ gene was constructed by inserting HindIII-BspH1 fragment containing rrnB T1T2 terminator from pX1(-ATG) into pZ1. pNH1Psi(SL1234) containing lacZ gene which is flanked by HIV psi(Ψ) nucleotide sequence right before the starting codon was constructed by inserting 4 stem-loop structure-containing psi(Ψ) fragment from pLLIII into pNH1 constructed above(see: Figure 1). And then, NC protein-

expressing pJC1 vector which is constructed by the present inventors(see: Ji Chang You and Charles S. McHenry, J. Biol. Chem., 268:16519-16527, 1993) and pNH1Psi(SL1234) constructed above are cotransformed into *E.coli*. That is, vectors pJC1 and pNH1Psi(SL1234) are cotransformed into *E.coli* by electroporation, followed by selecting antibiotics resistant transformant in medium containing antibiotics. The transformant was named *E.coli*/pNH1Psi(SL1234), and deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea), under an accession(deposition) No. KCCM-10194 on March 31, 2000. The transformant prepared above has a characteristic that β -galactosidase reporter gene expression is down regulated due to the interaction of psi(Ψ) nucleotide sequence with HIV NC protein, thereby it can be used for screening of HIV packaging inhibitors. For instance, the culture of said transformant is treated with putative compounds or compositions of HIV inhibitors, and then the expression level change of β -galactosidase in culture is determined. Therefore, the transformant of the invention can be used for screening of HIV packaging inhibitors which block the binding of HIV NC protein to HIV psi(Ψ) nucleotide sequence.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Construction of pNH1(SL1234)

A plasmid containing β-galactosidase reporter gene(SEQ ID NO: 1) was constructed.

Example 1-1: Construction of pNH1

LacZ gene fragment obtained by PstI (Boeringer Mannheim, Germany) digestion of pUS935(see: Dellagostin, O. A. et al., Microbiology, 141:1785-1792, 1995) was inserted into pSE280(Invitrogen, U.S.A.) to obtain pX1. In order to prevent translation from starting at ATG which is located upstream of the multicloning site of LacZ fragment, upstream ATG was removed by cutting pX1 with NcoI, mung bean exonuclease(Boehringer Mannheim, Germany) treatment, Klenow enzyme treatment to create blunt end and then ligating to make pX1(-ATG). Figure 1a is a schematic representation of construction strategy of pX1(-ATG). pZ1 was made by substituting ampicillin resistance gene of pX1(-ATG) with a kanamycin resistance gene, that is, by ligating the lacZ gene-containing MscI fragment from pX1(-ATG) and Kan^r gene-containing AflIII-StuI fragment from pZerO-2(Invitrogen, U.S.A.). Then, pNH1 was made by inserting rrn T1T2 terminator-containing HindIII-BspHI fragment from pX1(-ATG) into the ScaI site of pZ1. Figure 1b is a schematic representation of construction strategy of pNH1.

Example 1-2: Construction of pNH1Psi(SL1234)

pLLIII(University of Colorado, Health Sciences Center, Charles S. McHenry), a plasmid containing the 5' long terminal repeat(LTR) of HIV-1, was digested with SacI and MseI and then treated with Klenow enzyme to obtain a fragment containing HIV psi(Ψ) nucleotide sequence(named "SL1234", SEQ ID NO: 2) which contains 4 stem-loop structures.

SL1234 was digested with MaeI and then treated with Klenow fragment to obtain SL12(SEQ ID NO: 3). pNH1Psi(SL1234) or pNH1Psi(SL12) was made by inserting SL1234 or two fragments of SL12 into the BstEII site which is located upstream of lacZ gene in pNH1, respectively. Then, pNH1rePsi(SL1234) or pNH1rePsi(SL12) containing HIV psi(Ψ) nucleotide sequence in reverse orientation was made

to be used as a control vector. DNA bands of 221bp for right orientation and 152bp for reverse orientation were identified by 2% agarose gel electrophoresis.

Primers, U2 and L3 pair or U2 and L4 pair, were used for amplification of SL23(SEQ ID NO: 4) or SL234(SEQ ID NO: 5), respectively, and their nucleotide sequences are as follows.

primer U2 (SEQ ID NO: 6)
5'-GGG**GGTGACCTTTAAA**AGCAAGAGGCGAGGG-3'
primer L3 (SEQ ID NO: 7)
5'-GGG**GGTGACC**CTCTCCTTCTAGCCTCCG-3'
primer L4 (SEQ ID NO: 8)
5'-GGG**GGTGACC**GACGCTCTCGCACCCGTCTCT-3'

In order for easier insertion of psi(Ψ) fragment into BstEII site in pNH1, BstEII site(boldface) was inserted into the each primer, and DraI site(boldface) was inserted into the primer U2 to identify orientation. Also, point mutation(underlined), T to G, was introduced into primer L4 to prevent translation from starting in the psi(Ψ) sequence. PCR was performed in a 100 $\mu\ell$ of reaction mixture containing 10mM Tris-HCl, pH 7.5, 1.5mM $MgCl_2$, 50mM KCl, 1mM dNTP, 10μ M of each primer, 1 μg of pLLIII and 2.5 unit of Taq polymerase(Boehringer Mannheim, Germany) with preincubation at 94℃ for 5min, 30 cycles of denaturation at 94℃ for 1min, primer annealing at 60℃ for 2min, and extension at 72℃ for 2min, and then additional extension at 72℃ for 10 min. For SL23 insertion, DNA bands of 213bp for right orientation and 146bp for reversed orientation were identified by 2% agarose gel electrophoresis following digestion of each plasmid with DraI. For SL234 insertion, DNA bands of 234bp for right orientation and 146bp for reversed orientation were identified by 2% agarose gel electrophoresis following digestion of each plasmid with DraI.

SL34 fragment obtained by digestion of PCR product,

SL234, with RsaI and BstEII, was treated with Klenow fragment, and then inserted into the BstEII site of pNH1 to make pNH1Psi(SL34) and pNH1rePsi(SL34) as a control. DNA bands of 40bp for right orientation and 56bp for reversed orientation were identified by 4% agarose gel electrophoresis following double-digestion of each plasmid with BsmAI and XmnI.

In order to use as a negative control, that is, to see whether NC protein or Gag protein can interact with nucleotide sequence which is non-homologous to psi(Ψ), pNH1MCS was made by inserting 75bp MCS(multicloning site) fragment, which has no ATG starting codon, obtained by digestion of pSE280 with MaeI and subsequent Klenow treatment, into the BstEII site of pNH1. DNA band of 229bp was identified by 2% agarose gel electrophoresis following RsaI digestion. Figure 1c is a schematic representation of construction strategy of pNH1Psi or pNH1rePsi.

Example 2: Expression of HIV NC protein or HIV Gag protein

NC protein-expressing plasmid, pJC1, which was made and disclosed by the present inventors(see: Ji Chang You, and Charles S. McHenry, J. Biol. Chem., 268(22):16519-16527, 1993) or Gag protein-expressing plasmid, pTrcHisGag, was transformed into *E.coli* strain JM109(Promega Co., U.S.A.) by electroporation: to obtain competent cells, overnight culture of JM109 started from single colony was inoculated into 200ml of fresh LB medium(Luria-Bertani, Difco, U.S.A.) with 1:100 dilution and cultivated to OD 0.5-0.7, and then cells were centrifuged at 4000 g for 10 min., followed by 4 times of washing process which was resuspending cell pellet in cold 10%(v/v) glycerol and centrifugation at 4000 g for 10 min. After being resuspended in 1ml, 20μl of competent cells were mixed with 1-100 ng of DNA, transferred to 0.2 cm gap electroporation cuvette, and transformed in *E.coli* pulser(Bio-Lab, Heracules, CA, U.S.A.) by applying 2.5kV electric shock. The transformant was incubated overnight

in LB medium containing 100 μg of ampicillin, and then subcultured in fresh LB medium with 1:100 dilution. At the early period of logarithmic phase of growth, 1mM of isopropyl-β -D-thiogalactopyranoside(IPTG, Roche Diagnostics, Germany) was added and the proteins were induced for 3 hours. The liquid culture of transformant was sampled at each hour and centrifuged at 10,000 g for 1 min. The cell pellet was resuspended in 20 μl of gel loading buffer(50mM Tris-HCl, pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol), and incubated for 5 min at 100℃ to disrupt cell wall. After centrifugation at 10,000 g for 30 min, each supernatant was subject to 15% SDS-PAGE at 120V for 2 hours. The gel was stained in staining solution(0.25% Coomassie Brilliant Blue, 45% methanol and 10% glacial acetic acid) for 20 min and then destained in destaining solution(30% methanol and 10% acetic acid) for 2 hours. Although HIV NC protein(7kD) band did not appear without IPTG induction, the protein band at 7kD position was getting thicker after IPTG induction as time went by. Also, HIV Gag protein(55kD) band appeared at about 55kD position after IPTG induction.

Example 3: Cotransformation and measurement of β -galactosidase expression

In order to measure the effect of interaction between HIV psi(Ψ) nucleotide sequence and HIV NC protein or Gag protein on β -galactosidase expression level, pJC1 and each of pNH1Psi plasmids or pTrcHisGag and each of pNH1Psi plasmids were cotransformed into *E.coli* JM109 by electroporation, respectively. To exclude the possibility of plasmid copy number effect and to confirm the expression of lac repressor, JM109 was cotransformed with pSE380(Invitrogen, U.S.A.) as a control plasmid and each of pNH1Psi plasmids. Each cotransformant was selected on LB agar plate containing 100 μg/ml ampicillin and 40 μg/ml kanamycin.

To measure the expression of β -galactosidase in the cotransformants, β -galactosidase liquid assay was performed for each cotransformant more than 3 times. Each cotransformant was cultured in LB medium overnight, and the culture broth was inoculated into 5ml of fresh LB medium with 1:10 dilution. At the early stage of logarithmic phase of growth, 1ml aliquot of culture broth was taken and kept on ice, and then incubation was continued for 3 hours to allow expression of NC protein, Gag protein and β -galactosidase after 1mM IPTG was added for induction of β -galactosidase. And then, 1ml aliquot of culture broth was sampled each hour and kept on ice. Cell density was measured at A_{600} with enzyme-linked immunosorbent assay reader(ELISA reader, Dynatech, U.S.A.). For measurement of β -galactosidase activity, 50 ㎕ aliquot of each cotransformant culture broth was mixed with 450 ㎕ aliquot of Z buffer(60mM $Na_2HPO_4 \cdot 7H_2O$, 40mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4 \cdot 7H_2O$, 50mM β -mercaptoethanol). And then, cell wall was disrupted by addition of 20 ㎕ of chloroform and 10 ㎕ of 0.1% SDS followed by stirring for 30 seconds and incubating at 28℃ for 5min. To the cell lysate, 100 ㎕ of o-nitrophenyl-1-thio-β -D-galactopyranoside(ONPG, 4mg/ml, Sigma, U.S.A.) was added and the mixture was incubated at room temperature until yellow color developed. When yellow color was fully developed, the reaction was stopped by adding 200 ㎕ of 1M Na_2CO_3, and the mixture was clarified by centrifugation at 10,000 g for 30 min. Optical density(OD) of supernatant was measured at 420nm and 550nm. The activity(units) of β -galactosidase was quantitated by following equation:

Enzyme unit of β -galactosidase

$$= 1{,}000 \times (OD_{420} - 1.75 \times OD_{550}) / (t \times OD_{600})$$

wherein,

t is reaction time(minutes); and,

1.75 is a constant.

In order to determine if NC protein or Gag protein affect the β-galactosidase expression from the plasmid without psi(Ψ) sequence, *E.coli* JM109 was cotransformed with the precursor plasmid, pNH1(containing lacZ gene, no psi(Ψ) sequence) and pJC1(nucleocapsid), pNH1 and pTrcHisGag(Gag protein), or pNH1 and pSE380(control), respectively, and then, β-galactosidase activity was measured as described above. Figure 2 is a graph showing the expression of β-galactosidase in JM109 cotransformed with each of pJC1 expressing nucleocapsid protein, pTrcHisTag expressing Gag protein, or pSE380 as a control plasmid, and pNH1. As shown in Figure 2, the level of β-galactosidase expression in cotransformant expressing NC protein or Gag protein was not different from that in cotransformant expressing no other protein(pSE380) after 3 hour-induction of β-galactosidase with IPTG, therefore, it was demonstrated that NC protein or Gag protein had no effect on the expression of β-galactosidase in cotransformants.

In order to demonstrate the specificity of the interaction between HIV NC protein and HIV psi(Ψ) sequence or Gag protein and HIV psi(Ψ) sequence, β-galactosidase expression was measured in cotransformants containing each of pSE380, pJC1 or pTrcHisGag and each of pNH1MCS(multiple cloning site, MCS, as a non-homologous sequence to HIV psi(Ψ) nucleotide sequence) or pNH1Psi(SL1234, HIV psi(Ψ) nucleotide sequence), respectively. Figure 3a is a graph showing the effect of interaction between HIV nucleocapsid protein or Gag protein and non-homologous sequence to psi(Ψ) on the expression of β-galactosidase after induction with IPTG in JM109 cotransformed with each of pJC1, pTrcHisTag or pSE380 and pNH1MCS. Figure 3b is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and homologous psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1Psi(SL1234),

respectively. As shown in Figure 3a, the level of β-galactosidase in cotransformants containing non-homologous sequence(pNH1MCS) to psi(Ψ) and a plasmid expressing NC protein or Gag protein was not different from that in cotransformant containing pHN1MCS and control plasmid, pSE380. Meanwhile, as shown in Figure 3b, when transformed together with pNH1psi(SL1234), the level of β-galactosidase in cotransformant expressing Gag protein was reduced by 15% compared to that in cotransformant containing control vector, pSE380, moreover, cotransformant expressing NC protein showed more than 90% reduction in β-galactosidase expression compared to the control cotransformant.

Therefore, it was demonstrated that β-galactosidase expression in cotransformant was reduced by HIV NC protein in the presence of HIV psi(Ψ) nucleotide sequence and the reduction was caused by a specific interaction between HIV NC protein and HIV psi(Ψ) nucleotide sequence.

In order to identify which portion of psi(Ψ) nucleotide sequence is responsible for the specific interaction with NC protein, each of plasmids containing various portions of stem-loop structures of psi(Ψ) nucleotide sequence were cotransformed with NC- or Gag-expressing vector into *E. coli* JM109, respectively, and then β-galactosidase activity of each cotransformant was measured. Figure 4 is a graph showing the effect of specific interaction between HIV NC protein or Gag protein and portions of psi(Ψ) sequence on the expression of β-galactosidase in *E. coli* JM109 cotransformants. *E. coli* JM109 was cotransformed with each of pJC1, pTrcHisGag, or pSE380 and each of plasmids containing various portion of stem-loop structures of psi(Ψ) nucleotide sequence, pNH1Psi(SL1234), pNH1Psi(SL234), pNH1Psi(SL34), pNH1Psi(SL23), or pNH1Psi(SL12), respectively. In Figure 4, horizontal axis indicates the cotransformants containing each of pJC1, pTrcHisGag, or pSE380 and each of psi(Ψ)

structure-containing plasmids(lane 1 is control plasmid, pNH1; lane 2, a plasmid containing non-homologous sequence, pNH1MCS; lane 3, pNH1Psi(SL1234); lane 4, pNH1Psi(SL234); lane 5, pNH1Psi(SL34); lane 6, pNH1Psi(SL23); and, lane 7, pNH1Psi(SL12)), and vertical axis indicates the activity of β -galactosidase. The results were statistically analyzed by Student's test and * indicates $p<0.05$. As shown in Figure 4, HIV NC protein reduced the expression of lacZ gene more effectively when psi(Ψ) sequence is present in cell than HIV Gag protein did. When pNH1Psi(SL1234) containing full length psi(Ψ) nucleotide sequence and pJC1 were cotransformed into pJM109, the reduction of lacZ expression was most significant, and the degree of reduction was decreased in the order of SL34, SL234, SL23, and SL12. The sequence of SL4 appears to be more important than other stemp-loop structures for the specific interaction of psi(Ψ) sequence with NC protein which resulted in the reduction of lacZ gene expression.

Therefore, these results demonstrated that all of 4 stem-loop structures in psi(Ψ) sequence was required for the most significant reduction of β -galactosidase expression, and sequence of SL4 was the most important portion in psi(Ψ) sequence for NC protein binding. And, the degree of reduction of lacZ gene expression in pNH1Psi(SL234) and pNH1Psi(SL34) which were point-mutated to avoid translation starting from SL4 area was similar to that in pNH1Psi(SL1234) without point mutation. Thus, the transformant was named as *E.coli*/pNH1Psi(SL1234), which showed the most significant reduction of β -galactosidase expression by specific interaction of psi(Ψ) sequence with NC protein. *E.coli*/pNH1Psi(SL1234) was deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea), under an accession(deposition) No. KCCM-10194 on March 31, 2000. The results obtained from the above Examples were summarized in Table 1 below.

Table 1: Level of β -galactosidase expression depending on kinds of reporter vectors

Reporter Vector		Enzyme units of β -galactosidase after 3-hour induction					
		pSE380 (Control)		pJC1 (expressing NCp7)		pTrcHisGag (expressing Gag)	
pNH1	-	302±19*	100%	295±28.7	98%	298±20.8	96%
pNH1MCS	75bp	296±24.5	100%	234±40.5	80%	296±44.5	100%
pNH1Psi (SL1234)	SL1234	119±22	100%	8±2.2	6.7%	103±23	86.5%
pNH1Psi (SL234)	SL234	308±42.5	100%	87±25.5	28%	140±44	76%
pNH1Psi (SL34)	SL34	85±14	100%	11±2.6	13%	68±12	80%
pNH1Psi (SL23)	SL23	298±55.5	100%	112±14	38%	214±22.5	72%
pNH1rePsi (SL12)	SL12	186±32	100%	107±3.5	58%	158±24	85%
pNH1rePsi (SL1234)	reSL1234	87±21	100%	51±10	59%	85±17	98%
pNH1rePsi (SL234)	reSL234	185±37	100%	78±5	42%	140±30.5	76%
pNH1rePsi (SL34)	reSL34	311±29	100%	186±	60%	307±30	99%
pNH1rePsi (SL23)	reSL23	305±43	100%	131±19	43%	280±18	92%
PNH1Psi (SL12)	reSL12	86±21	100%	64±11	75%	86±19.5	100%

As a result, it is a characteristic of the transformant to be reduced in β -galactosidase reporter gene expression due to the specific interaction between psi(Ψ) nucleotide sequence and HIV NC protein. Therefore, the transformant of the invention can be used for screening of HIV packaging inhibitors which block the binding of HIV NC protein to HIV psi(Ψ) nucleotide sequence, by treating the culture broth of *E.coli* JM109(KCCM-10194) with putative compounds or compositions of HIV inhibitors and measuring the degree of change in β -galactosidase expression.

As clearly illustrated and demonstrated above, the present invention provides a microorganism cotransformed with a gene expressing HIV nucleocapsid protein and a plasmid vector containing HIV Psi(Ψ) gene and β -

galactosidase reporter gene, and a method for screening HIV packaging inhibitors employing the said transformant. The invented method comprising the steps of culturing the said transformant, treating it with putative compounds or compositions of HIV inhibitors, and measuring the degree of change in β -galactosidase expression in the culture, can be practically applied in screening HIV packaging inhibitors by which the interaction between HIV nucleocapsid and HIV psi(Ψ) sequence is blocked.

AMENDED CLAIMS

[received by the International Bureau on 01 August 2001 (01.08.01);
original claims 1and 2 replaced by amended claims1-20(3pages)]

1. A microorganism cotransformed with a plasmid vector containing a gene expressing HIV nucleocapsid protein, and a plasmid vector containing HIV psi(ψ) gene and β -galactosidase reporter gene.

2. The microorganism of claim 1 wherein the plasmid vector containing a gene expressing HIV nucleocapsid protein is pJC1.

3. The microorganism of claim 1 wherein the HIV psi(ψ) gene is SL1234(SEQ ID NO: 2).

4. The microorganism of claim 1 wherein the HIV psi(ψ) gene is SL234(SEQ ID NO: 5).

5. The microorganism of claim 1 wherein the HIV psi(ψ) gene is SL23(SEQ ID NO: 4).

6. The microorganism of claim 1 wherein the HIV psi(ψ) gene is SL12(SEQ ID NO: 3).

7. The microorganism of claim 1 wherein the β -galactosidase reporter gene is SEQ ID NO: 1.

8. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL1234).

9. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL234).

10. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL23).

11. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(ψ) gene and β-galactosidase reporter gene is pNH1Psi(SL12).

12. *E.coli* JM109(KCCM-10194) cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL1234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

13. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

14. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL23) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

15. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL12) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

16. A microorganism transformed with a vector pNH1Psi(SL1234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

17. A microorganism wherein both a plasmid vector containing a gene coding for HIV nucleocapsid protein and a plasmid vector containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1) are integrated into a chromosome.

18. A method for screening HIV packaging inhibitors which comprises the steps of:

(i) culturing the cotransformed microorganism of claim 1;

(ii) treating the said cotransformed microorganism with putative compounds or compositions of HIV inhibitors; and,

(iii) measuring the degree of change in β-galactosidase expression in the culture.

19. The method of claim 21 wherein the cotransformed microorganism is *E.coli* JM109(KCCM-10194).

20. Use of the cotransformed microorganism of claim 1 for HTS(High Throughput Screening) of HIV inhibitors.

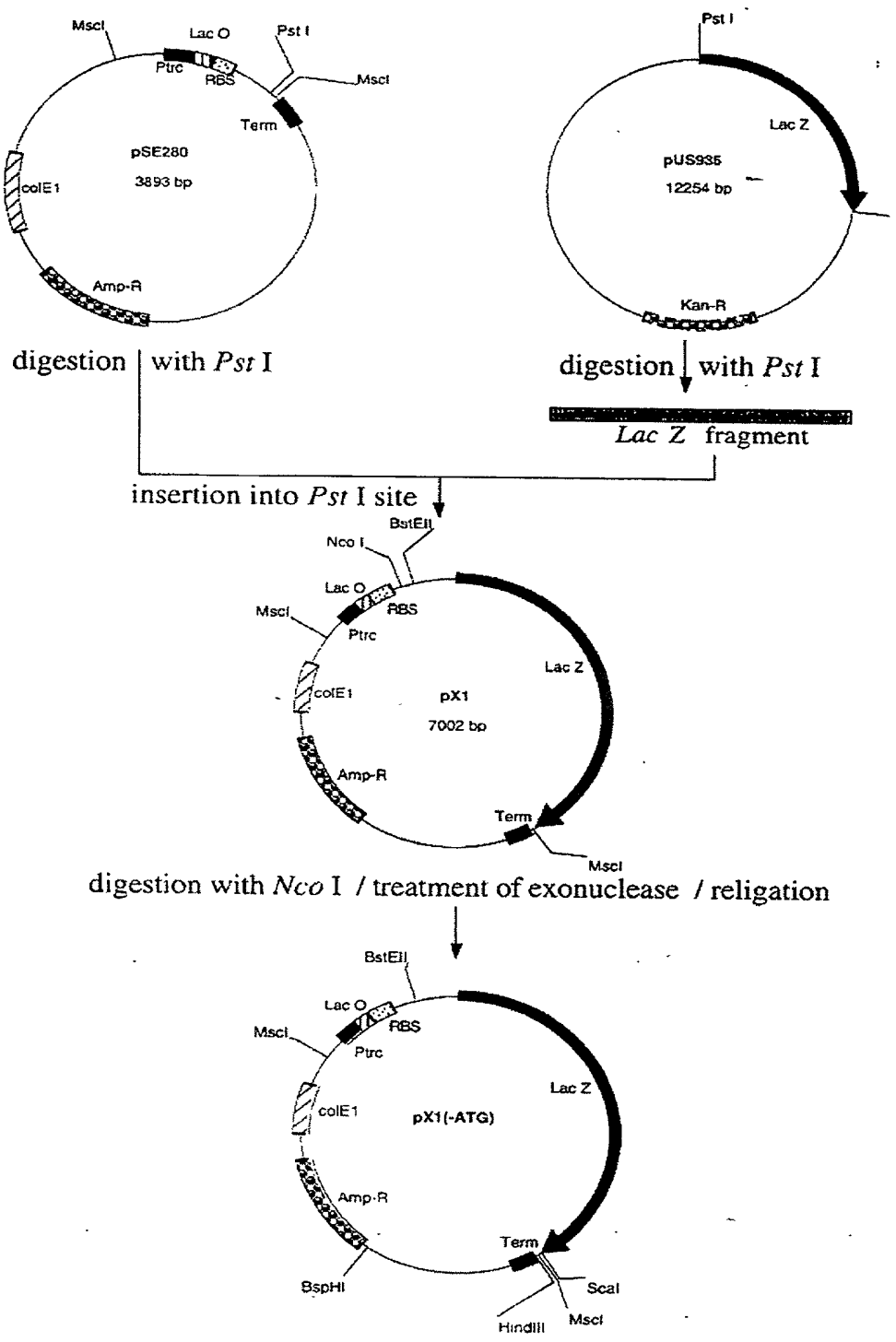
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Fig. 1a

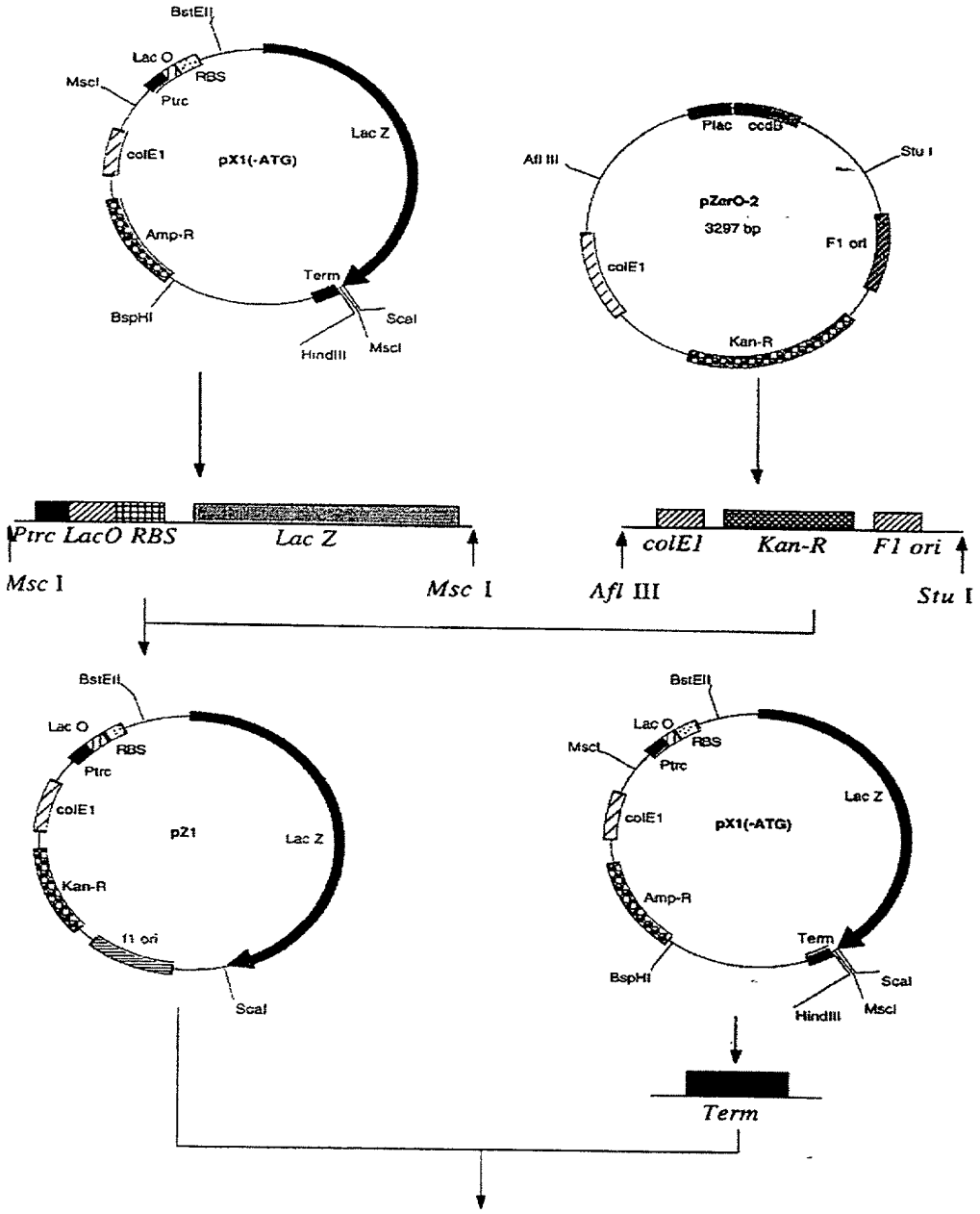
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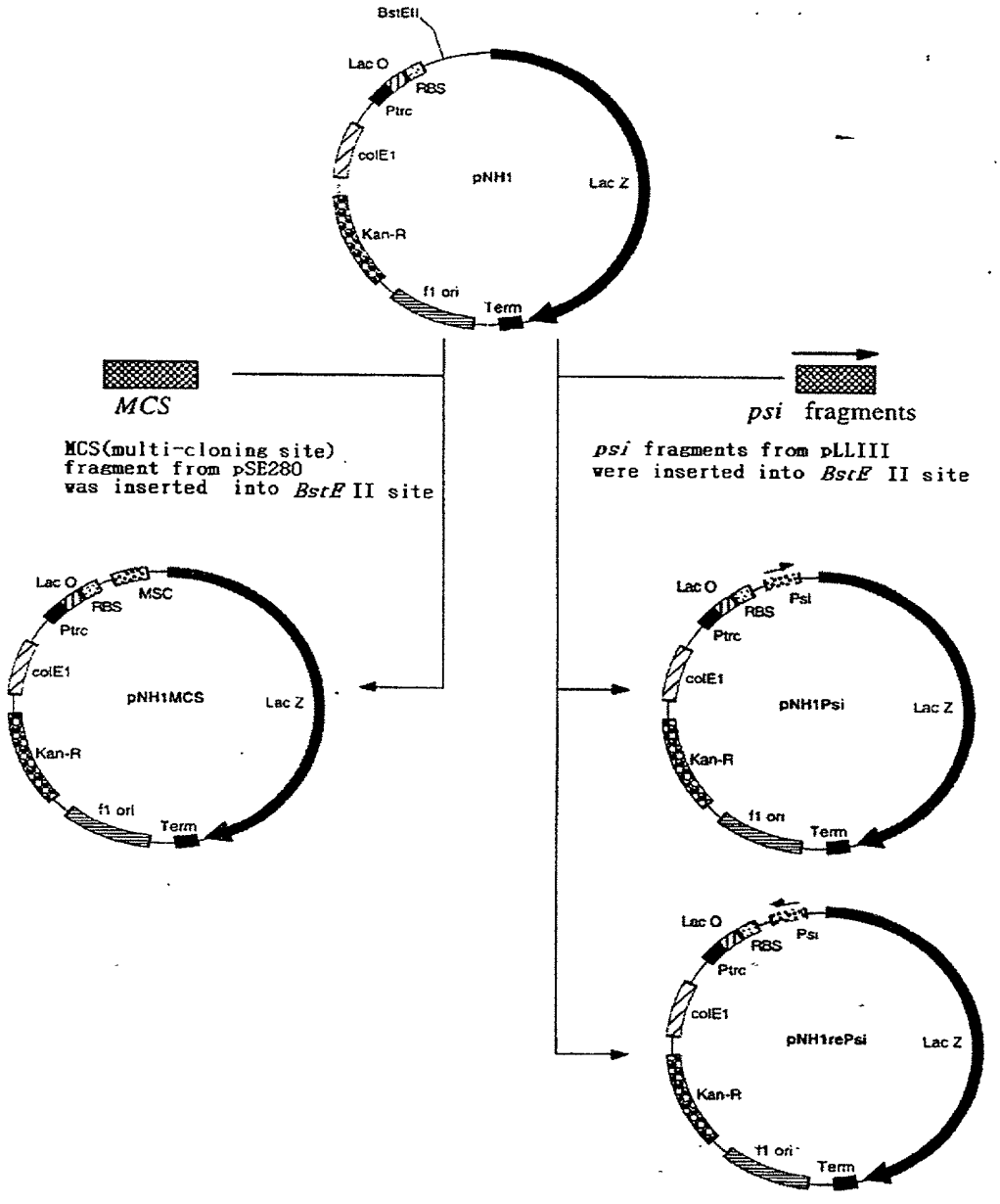
Fig. 1b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was inserted into BstE II site
psi fragments
psi fragments from pLLIII
were inserted into BstE II site
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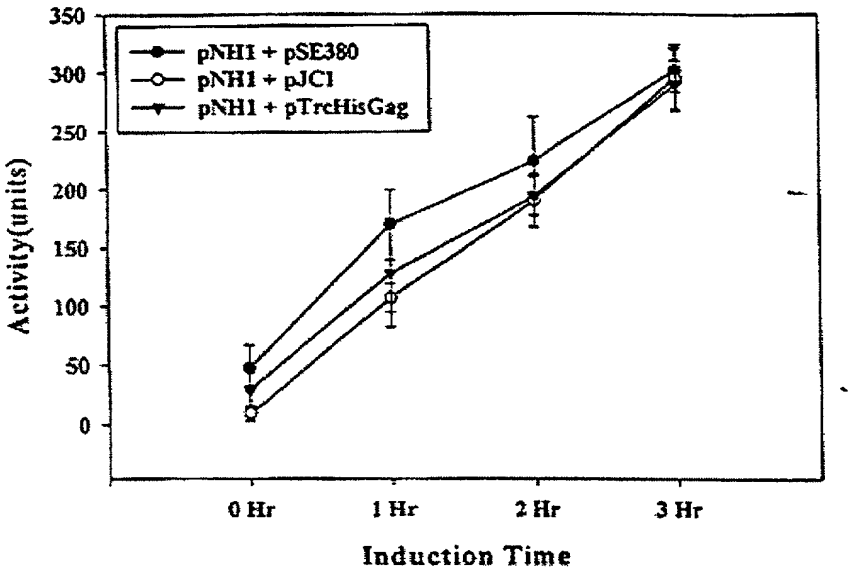
pNH1MCS
Psi
pNH1Psi
pNH1rePsi

Fig. 1c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pNH1 + pTrcHisGag
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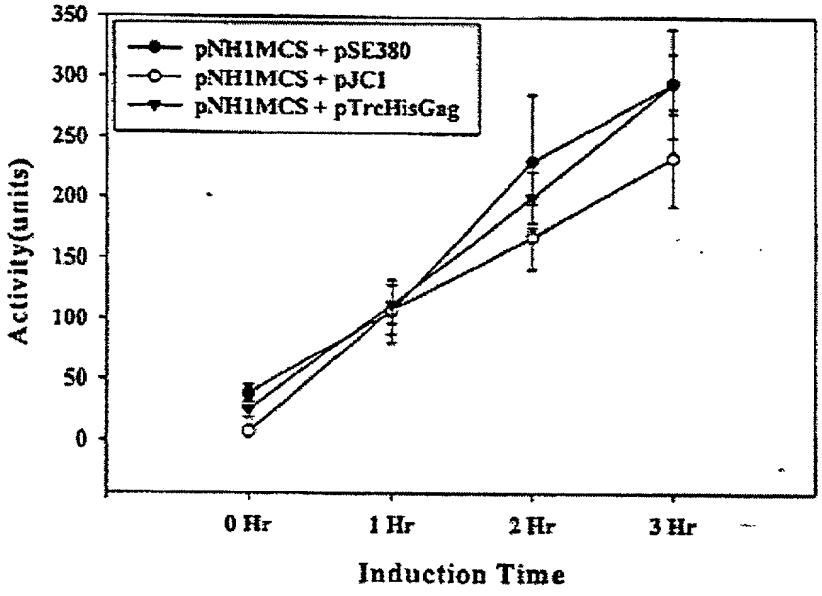
2 Hr
3 Hr
Induction Time

Fig. 2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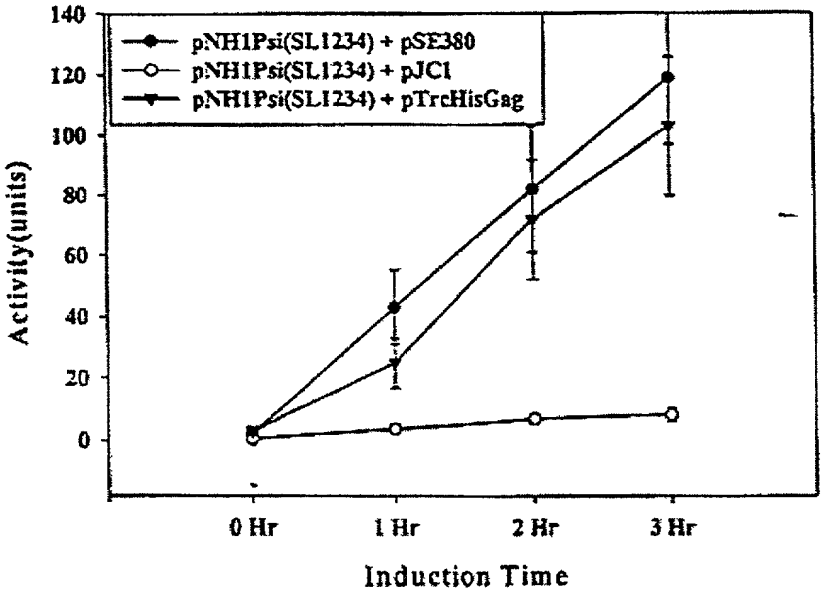
0
0 Hr
1 Hr
2 Hr
3 Hr
Induction Time

Fig. 3a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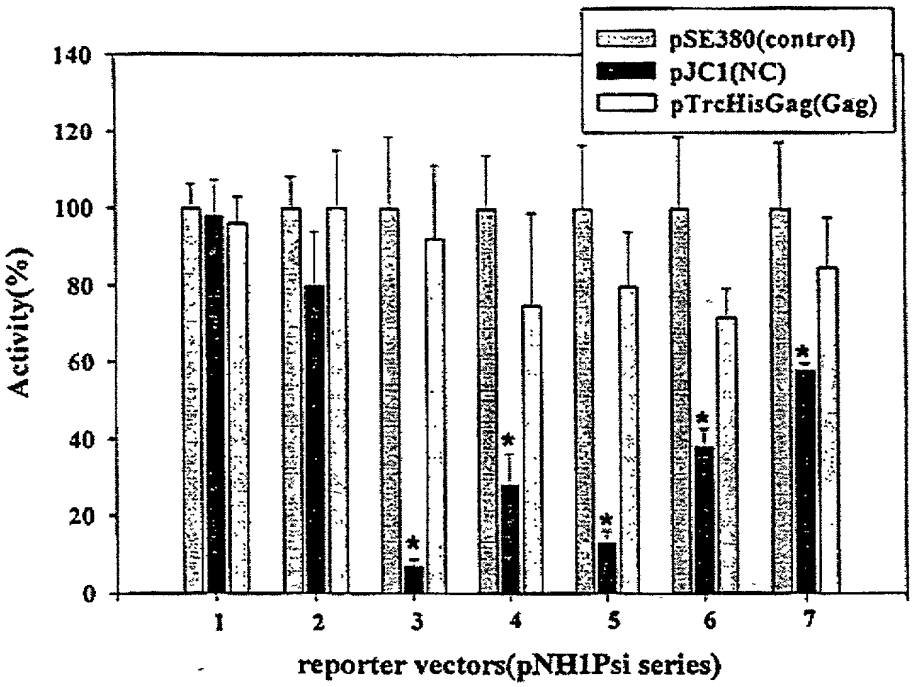
2 Hr
3 Hr
Induction Time

Fig. 3b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pTrcHisGag(Gag)
Activity(%)
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120
100
80
60
40
20
0
1
2
3
4
5
6
7
reporter vectors(pNH1Psi series)

Fig. 4

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

As below named inventors, we hereby declare that:

Our residences, post office addresses and citizenships are as stated below next to our names. We believe we are the original, first and joint inventors of the subject matter which is claimed for and which a patent is sought on the invention entitled:

"A TRANSFORMANT FOR SCREENING OF INHIBITORS FOR HUMAN IMMUNODEFICIENCY VIRUS"

the specification of which is attached hereto.

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by us on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119

COUNTRY (if PCT Indicate PCT)	APPLICATION NO.	DATE OF FILING (day/month/year)	PRIORITY CLAIMED
Korea	2000-0018489	08/04/00	YES
PCT	PCT/00/01173	18/10/00	NO

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, Morris Relson #15,108, Gordon D. Coplein ~~#19,165~~, William F. Dudine, Jr. #20, 569, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Beverly B. Goodwin #28,417, Adda C. Gogoris #29,714, Martin E. Goldstein #20,869, Bert J. Lewen #19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194, David R. Francescani #29,159, Robert C. Sullivan, Jr., #30,499

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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Sequence Listing

<110> YOU, Ji-Chang

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